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III. BETA-GLUCOSIDASE CROSSLINKING FOR STABILITY AND ENZYME RECYCLE

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INTRODUCTION

A major problem encountered in carrying out cellulose digestion at high temperatures is the low thermal stability of commercial beta-glucosidase preparations, compared with the thermal stability of the other components of the cellulase complex. At temperatures above 55-60 C, rapid inactivation of the beta-glucosidase leaves the other components -- cellobiohydrolase in particular -- subject to inhibition by large accumulations of cellobiose. Provision of a more thermostable beta-glucosidase component would therefore result in substantial increase in efficiency for the process as a whole.

[redacted] have seen the development [redacted] chemical reagents that are capable of forming cross-links between either the same, or different types of protein functional groups. Applications of these reagents in protein chemistry have included the attachment of enzymes to insoluble supports (Hornby and Goldstein, 1976), the insolubilization of protein crystals (Quicho, 1976; and Lee et al, 1986), and mapping of the subunit structure of multimeric enzymes (Pfeuffer et al, 1985). In an application of great interest to those of us concerned with the production of soluble enzymes of high thermal stability, Woodward et al, (1981), reported that treatment of a commercial enzyme mixture with glutaraldehyde resulted in a substantial increase in the resistance to thermal denaturation shown by the beta-glucosidase activity present in the mixture. Since the study by Woodward was carried out using a commercial mixture containing a variety of chemical species other than the beta-glucosidase (polysaccharides, other proteins, etc.), one should consider the possibility that the observed stabilization might have been due to attachment of the beta-glucosidase to some of these other species, in addition to the possibility that stabilization was due to inter- or intramolecular crosslinking of the beta-glucosidase itself. In order to answer this and other questions concerning the general principles that control stabilization of enzymes by crosslinking reagents, we decided to extend the studies of Woodward by (1) purifying the beta-glucosidase activity and using the purified enzyme for crosslinking studies, (2) following the thermal inactivation kinetics for both native and modified enzyme over longer periods of time than those reported in the earlier work, (3) applying other crosslinkers that have only recently become available, and (4) developing effective methods for separating stabilized from non-stabilized enzyme, thus making possible the kinetic, chemical, and physical study of the stabilized enzyme in isolation, rather than as component(s) of a mixture.

EXPERIMENTAL, RESULTS AND DISCUSSION

The increase in thermal stability of Aspergillus niger beta-glucosidase upon treatment with the bifunctional crosslinking agent dimethlysuberimidate (DMS) has been compared with the enhancement in thermal stability observed upon treatment with glutaraldehyde. The following figure illustrates the decay in enzyme activity shown by various enzyme preparations when pre-

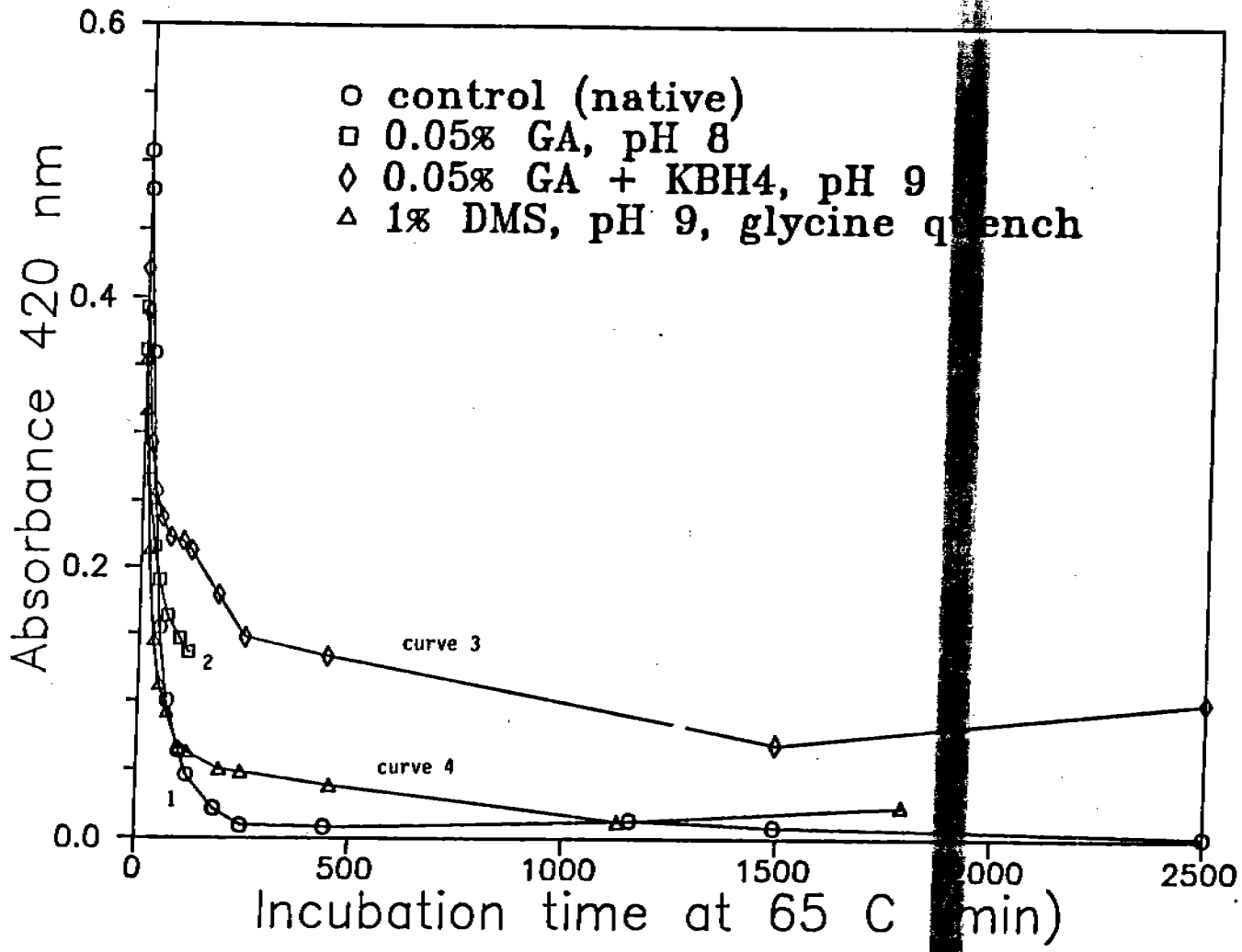

Figure 1. Effect of Glutaraldehyde Treatment on Stability of A. niger beta-glucosidase.

incubated for the indicated times at 65 C (pH 5, 10 mM acetate, 0.1 M NaCl; buffer A) prior to assay at 45 C versus p-nitrophenyl-beta-D-glucoside. Curve 1 shows that when native Aspergillus beta-G I is diluted to 0.04 ug/mL in buffer A, and preincubated at 65 C for the indicated times, the activity (measurable by subsequent assay at 45 C) decreases with a half-life of approximately 30 minutes, so that after 3 hours exposure to 65 C, less than 5% of the original activity remains, with the activity decreasing essentially to zero by 5 hours exposure. In contrast, a sample of enzyme treated with 0.05% glutaraldehyde (pH 8 in 0.05 M phosphate, 25 minutes 22 C) retains more than 15% of the activity of the native enzyme after 3 hours at 65 C (curve 2).

Enzyme treated with the same concentration of glutaraldehyde for approximately the same duration, but at pH 9 in 0.2 M borate, with a subsequent borohydride reduction step, shows even greater stabilization (curve 3). In this case, the stability of the enzyme at 65 C was monitored for much longer times than in the previous experiments, and the enzyme solution was found to retain almost 13% of the original (pre-crosslinking) activity even after 43 hours exposure to 65 C, with [illegible] between 24 and 43 hours. One rationalization of the shapes of this curve is that the glutaraldehyde-treated enzyme is composed of at least two different sub-populations of enzyme molecules. One, which we will call "population I", is composed of molecules that are inactivated with essentially the same half-life as the native enzyme (ca. 30 minutes at 65 C). The initial 3-4 hours of the decay curve is dominated by the inactivation of Population I, which represents some 70% of the activity found in the treated enzyme prior to exposure at 65 C. The remainder of the activity in the treated enzyme is due to "Population II" enzyme molecules which have half-lives too long to be measured by the present experiments but which are at least on the order of days, as opposed to 30 minutes for the native enzyme,

One obvious goal for future research is to search for reaction conditions that will shift the resultant mixture in favor of "Population II". A simple increase in glutaraldehyde concentration does not appear to be the answer; at pH 8, at least, a 10-fold higher concentration of glutaraldehyde produces less, not more, stabilization. This result may indicate that the "Population I" enzyme, which resembles native enzyme in stability, does not represent enzyme that has not reacted with crosslinker, but rather enzyme in which the available reaction sites (amino groups) have been consumed in reactions that do not enhance enzyme thermal stability.

Curve 4 represents the decay at 65 C of enzyme previously treated (at pH 9 in 0.2 M borate) with a crosslinker (DMS) that is chemically different from glutaraldehyde but which also reacts with enzyme amino groups. The overall pattern of activity loss/retention is similar to that seen with the glutaraldehyde-treated enzyme, but the degree of stabilization is less. This may be due to the side-reactions to which DMS is subject in aqueous solution (i.e., hydrolysis of the reactive group on one end of the molecule to produce a monofunctional reagent), which may result in monosubstitution rather than crosslinking of the enzyme. On the other hand, only three experiments have been run to date using DMS, and it is quite probable that reaction conditions for this reagent and this enzyme are not yet optimized.

The apparent production of an extremely stable sub-population of enzyme molecules by treatment with bifunctional amino-group reagents is very

encouraging, and points to at least two parallel approaches for future work: 1) optimization of reaction conditions to maximize Population II using the present reactions, and 2) finding different reactions, complementary to amino-group linking so that molecules in which productive amino group reactions have been preempted by non-stabilizing reactions, can be stabilized by the crosslinking of other groups.

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